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**Emended descriptions of *Bacillus sporothermodurans* and *Bacillus oleronius*
with the inclusion of dairy farm isolates of both species**

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24 *Bacillus sporothermodurans* is an industrially important micro-organism because of its ability to
25 produce endospores which resist ultra high temperature (UHT) and industrial sterilization
26 processes. It was described by Pettersson *et al.* (1996) based on seven genetically homogeneous
27 isolates all from UHT-milk. *Bacillus oleronius*, the closest phylogenetic neighbor of *B.*
28 *sporothermodurans*, was described by Kuhnigk *et al.* (1995), based on a single strain, isolated from
29 the hindgut of the termite *Reticulitermes santonensis*. A polyphasic study of a heterogeneous
30 collection of *B. sporothermodurans* and *B. oleronius* strains isolated from various sources and
31 geographic origins led to an emended description of both species. Additional data presented are the
32 results of fatty acids, quinones and/or cell wall analysis (polar lipids). DNA-DNA hybridizations
33 confirmed 3 subgroups of strains obtained after SDS-PAGE analysis of cellular proteins as *B.*
34 *sporothermodurans*. One named *B. sporothermodurans* strain (R-7489) was reclassified as a
35 *Bacillus fordii* strain. The phenotypic profiles of both species were rather heterogeneous,
36 sometimes different from the original descriptions and did not differ in a large number of
37 characters, although *B. oleronius* generally gave stronger reactions in its positive tests than did *B.*
38 *sporothermodurans*; the variable and weak reactions for both organisms with some substrates
39 blurred the distinction between both. However, differences in polar lipid, SDS-PAGE and
40 menaquinone profiles clearly allow distinction between the two species.

41 *Bacillus sporothermodurans* was described by Pettersson *et al.* (1996) as a species producing
42 highly heat-resistant endospores that may survive ultra high temperature (UHT) treatment of
43 milk. The spore resistance to UHT-conditions (typically 140°C for a few seconds) was proven
44 both with an isolate from UHT-milk (Huemer *et al.*, 1998) as with spores naturally occurring
45 in contaminated UHT-milk (Scheldeman *et al.*, 2006). Isolates of this species were also found
46 in sterilized milk and milk products such as UHT-cream, chocolate milk, evaporated milk and
47 reconstituted milk (Herman *et al.*, 2000). As a consequence, surviving spores may germinate
48 and grow in the consumer milk or milk product causing the technological problem of non-
49 sterility. This problem has been encountered by different dairy companies worldwide with the

remarkable observation that one clone (HRS-clone) was concerned in the majority of the cases;
 only a few German isolates were found to be different from this HRS-clone by a non-
 hierarchical three-dimensional scaling of molecular typing data (Guillaume-Gentil *et al.* 2002).
 In the same study, farm isolates of *B. sporothermodurans* isolated from raw milk, feed
 concentrate and silage were shown to be genetically very heterogeneous and different from the
 HRS-clone. These farm isolates were identified as *B. sporothermodurans* by a specific PCR
 and for an isolate from raw milk and from feed concentrate also by DNA-DNA hybridization
 (Scheldeman *et al.*, 2002). In the same study as well as in Vaerewijck *et al.* (2001), *Bacillus*
oleronius was isolated from raw milk and feed concentrate. Hitherto, this species had only
 been isolated from the hindgut of a termite and described on the basis of one isolate from this
 source (Kuhningk *et al.*, 1995).
 Because of the observed genetic heterogeneity, the original description of *B. sporothermodurans*,
 which was based on only a few genetically homogeneous UHT isolates belonging to the HRS-clone
 (Pettersson *et al.*, 1996), may no longer be adequate. One example is the upper growth limit reported
 by Pettersson *et al.* (1996) to be 50 °C for the type strain but shown to be as high as 55 °C for farm
 isolates (Scheldeman *et al.*, 2002). This prompted us to prepare an emended description of this
 industrially important organism as well as of its phylogenetic neighbour, *B. oleronius*, with the
 inclusion of genetically heterogeneous farm isolates for both species.

The *B. sporothermodurans* and *B. oleronius* strains used are listed in Table 1. They were
 grown on Brain Heart Infusion (BHI) (Oxoid) supplemented with Bacteriological agar no. 1
 (15 g l⁻¹) (Oxoid) and filter sterilized vitamin B₁₂ (1 mg l⁻¹) at 37 °C for 48 h for all analyses
 (unless otherwise indicated). The *B. sporothermodurans* specific PCR (BSPO-PCR) described
 by Scheldeman *et al.* (2002) was carried out on all strains listed above as well as on some
 additional *Bacillus fordii* strains described by Scheldeman *et al.* (2004). For determination of
 the G + C content of some selected strains, approximately 1 gram of biomass was harvested
 from agar plates and DNA was purified as in Logan *et al.* (2000) with the modifications as

outlined by Heyndrickx *et al.* (2004). The G + C content of DNA was determined by HPLC using further specifications given by Logan *et al.* (2000). DNA-DNA hybridizations were performed for strains *B. sporothermodurans* LMG 17668^T, LMG 19637 and R-6778, for *Bacillus shackletonii* LMG 18435^T and for *Bacillus acidicola* DSM 14745^T using a modification (Willems *et al.*, 2001) of the microplate method described by Ezaki *et al.* (1989) with a reassociation temperature of 37 °C. All strains (together with the additional *B. fordii* strains mentioned above), grown on supplemented BHI as outlined above, were subjected to SDS-PAGE analysis of whole cell proteins according to Pot *et al.* (1994). The SDS-PAGE data were collected and interpreted as described by Vauterin & Vauterin (1992). For gas chromatographic analysis of methylated cellular fatty acids (FAME), cells of the same strains were grown for 24h on supplemented BHI. Further analysis was performed as described by Dawyndt *et al.* (2006). All *B. sporothermodurans* and *B. oleronius* strains together with type (and reference) strains of unreactive or weakly reactive species were phenotypically characterized by the methods of Logan & Berkeley (1984): thirteen routine biochemical tests, and 48 tests for acid production from a range of carbohydrates were made using the API 20E and 50CHB kits (bioMérieux), following cultivation of *B. sporothermodurans* and *B. oleronius* strains on BHI agar supplemented with vitamin B₁₂ at 37 °C for 24 hours. Other phenotypic characters were determined as described by Heyrman *et al.* (2004), but using BHI or BHI agar supplemented with vitamin B₁₂ as the basal media. Phenotypic data for other species were obtained on Tryptic Soy Agar (TSA) (Oxoid). For peptidoglycan analysis, whole cell hydrolysates (4N HCl, 100°C, 16 hours) of *B. sporothermodurans* LMG 17668^T and *B. oleronius* LMG 17952^T were subjected to thin layer chromatography on cellulose plates using the solvent system of Rhuland *et al.* (1955). Quinones for both strains were determined as described by Groth *et al.* (1996). For polar lipid determination, cells of both type strains were grown for 24h at 37 °C in BHI broth (1liter flasks) supplemented with vitamin B₁₂ and analyses were carried out by the Identification Service of the DSMZ (Braunschweig,

Germany) by two-dimensional silica gel thin-layer chromatography and detection with appropriate reagents (Tindall *et al.*, 1990a & b).

Numerical analysis of SDS-PAGE patterns of whole cell proteins (supplementary Fig.) revealed three groups that all contain strains sharing at least 80 % similarity between their SDS-PAGE profiles. Groups 1 and 2 are subdivided at about 75 % similarity and group 3 branches at about 65 %. The first group is quite heterogeneous and contains the *B. sporothermodurans* isolates. This group can be further subdivided in three SDS-PAGE subgroups (I to III). The first subgroup I contains sixteen strains, including the type strain, the UHT-milk strains and strains from various other sources (sterilized milk, raw milk, soy and feed concentrate). The second subgroup II contains three Belgian strains (R-6777, R-6778 and R-6779) from different sources. The third subgroup III contains five Belgian strains isolated from feed concentrate. The second main SDS-PAGE group contains different strains previously described as *B. fordii* (Scheldeman *et al.*, 2004), as well as one strain (R-7489) labelled as *B. sporothermodurans*. The attribution of R-7489 to *B. sporothermodurans* is based on a positive reaction in the *B. sporothermodurans* specific PCR (BSPO-PCR) that was thought to be specific for *B. sporothermodurans* (Scheldeman *et al.*, 2002). However, the type strain of *B. fordii* (not described at the time) also gives a weak amplicon in the BSPO-PCR. On the basis of the performed analysis, R-7489 should be attributed to *B. fordii*. The third SDS-PAGE group in the SDS-PAGE clustering contains named *B. oleronius* strains and five strains putatively identified as *B. oleronius* by fatty acid analysis (Scheldeman *et al.*, 2004). SDS-PAGE thus confirmed the attribution of the latter strains to *B. oleronius*. The *B. oleronius* strains can be distinguished on the basis of their SDS-PAGE profile by a strong band in the region of 85 kDalton.

B. sporothermodurans M215^T (= LMG 17668^T) (U49078) shares 98.2% 16S rRNA gene sequence similarity with *B. oleronius* DSM 9356^T (= LMG 17952^T) (X82492), 97.6% with *B. acidicola* 105-2^T (= DSM 14745^T) (AF547209) and 97.2% with *B. shackletonii* LMG 18435^T (AJ250318). *B.*

128 *oleronius* DSM 9356^T (= LMG 17952^T) (X82942) shares 98% 16S rRNA gene sequence similarity
 129 with *B. acidicola* 105-2^T (= DSM 14745^T) and 96.6% with *B. shackletonii* LMG 18435^T
 130 (AJ250318). The species status of both *B. sporothermodurans* and *B. oleronius*, showing >97%
 131 16S rRNA gene sequence similarity with the above mentioned closely related species, was
 132 confirmed by low DNA-DNA hybridisation values (< 70%) as previously determined by
 133 Scheldeman *et al.* (2002) and Albert *et al.* (2005), and as determined in this study (Table 2). DNA-
 134 DNA hybridisation values determined by Scheldeman *et al.* (2002) and during this study for *B.*
 135 *sporothermodurans* strains LMG 17833 and LMG 19620 (both belonging to SDS-PAGE subgroup
 136 I), R-6778 (SDS-PAGE subgroup II) and LMG 19637 (SDS-PAGE subgroup III) with the *B.*
 137 *sporothermodurans* type strain LMG 17668^T confirmed the allocation of all three observed SDS-
 138 PAGE subgroups to the species *B. sporothermodurans* (hybridisation values > 70% (Table 2).
 139
 140 Fatty acid analysis did not allow a clear distinction between *B. sporothermodurans* and *B. oleronius*
 141 (Table 3). The *B. sporothermodurans* strains showed some more heterogeneity than the *B.*
 142 *oleronius* strains (as can be derived from the greater standard deviation values), but this
 143 heterogeneity could not be linked to the heterogeneity observed in SDS-PAGE analysis. Both
 144 species could clearly be differentiated from *B. fordii* by lower amounts of the dominant fatty acid
 145 iso-C_{15:0}, and higher amounts of anteiso-C_{17:0}.
 146
 147 *B. sporothermodurans* strains often grew poorly and benefited from the addition of vitamin B₁₂ to
 148 the medium. They also showed weak reactions in the phenotypic tests, and these reactions were not
 149 appreciably enhanced by the addition of vitamin B₁₂ to the test media. The characters were not only
 150 weak but also variable, so that the phenotypic data do not give a clearly distinctive profile that is
 151 characteristic for the species. *B. sporothermodurans* UHT milk isolates not only grew poorly but
 152 also sporulated poorly, yet their spores showed very high heat resistances (Scheldeman *et al.*,
 153 2006). This property of resistance to high temperatures decreased with subculture in the laboratory.
 Sporulation was enhanced by the addition of soil extract to the BHI medium supplemented with

154 MnSO₄ and vitamin B₁₂, but it is not known whether such spores have enhanced heat resistance.
155 Isolates from farm environments grew more readily than UHT milk isolates and their spores did not
156 show very high heat resistances (Scheldeman *et al.*, 2006). Strains of *B. oleronius* did not require
157 the growth medium to be supplemented with vitamin B₁₂, and all strains sporulated well. The
158 phenotypic profiles of the two species did not differ in a large number of characters, although *B.*
159 *oleronius* generally gave stronger reactions in its positive tests than did *B. sporothermodurans*; the
160 variable and weak reactions for both organisms with some other substrates blurred the distinction
161 between the two species (Table 4). The differentiation table includes the phylogenetic closest
162 relatives of both species, namely *B. acidicola* and *B. shackletonii*, as well as phenotypically related
163 species.

164 The type strains of *B. sporothermodurans* and *B. oleronius* contained both *meso*-diaminopimelic
165 acid as diagnostic diamino acid of the cell wall peptidoglycan. This means that both species show
166 the peptidoglycan type A1 γ (this can be concluded from the fact that *meso*-diaminopimelic acid has
167 been reported up to now only for the peptidoglycan type A1 γ and for three variations of the type A4
168 γ ; these variations of the type A4 γ have been found so far exclusively in the genera
169 *Brachybacterium* and *Dermabacter*, with which *B. sporothermodurans* and *B. oleronius* have no
170 relationship). The major polar lipids of the type strains of *B. sporothermodurans* and *B. oleronius*,
171 LMG 17668^T and LMG 17952^T were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG)
172 and phosphatidylethanolamine (PE), typical for the genus *Bacillus*. Minor amounts of an
173 aminophospholipid (PN) and an unidentified phospholipid could also be detected. However, both
174 species can be differentiated from each other based on the presence of three other unidentified
175 phospholipids (PL's) in *B. oleronius*.

176 The type strain of *B. sporothermodurans* contained the menaquinones MK-7, MK-8 and MK-6 (in
177 the ratio 51 : 45 : 1), while the type strain of *B. oleronius* contained MK-7, MK-8 and MK-9 (ratio
178 52.6 : 46.9 : 0.4).

Overall, it has been shown here that strains of *B. sporothermodurans* and *B. oleronius* are rather heterogeneous in phenotypic characteristics but belong to these respective valid species. Both species are difficult to differentiate from each other on the basis of biochemical and physiological characteristics. The identification of members of these species requires the use of a polyphasic approach based on well defined phenotypic, chemotaxonomic and/or genetic taxonomic properties as described above. An emended description of both species is warranted as given below, including new data on fatty acid analysis, diagnostic diamino acid, peptidoglycan type, polar lipids and menaquinone profile for *B. sporothermodurans*, and polar lipids and menaquinone profile for *B. oleronius*. Furthermore, some additions and modifications were made on cell morphology and spore properties and on biochemical characteristics based on API 50CHB and API 20E test results as contradictions were observed with the original species descriptions. Specifically for *B. sporothermodurans*, more of these tests (e.g. API 50 CHB) appeared positive in our hands.

Emended description of *Bacillus sporothermodurans*.

Bacillus sporothermodurans (spo.ro.ther.mo.du'rans, Gr. n. *spora*, seed, and in biology a spore; Gr. adj. *thermos*, warm, hot; L. part. adj. *durans*, resisting. N. L. part adj. *sporothermodurans*, with heat-resisting spores).

Aerobic, Gram-positive cells that usually occur as motile, thin rods in chains. Strains require vitamin B₁₂ for satisfactory growth. After 2d on Brain Heart Infusion (BHI) agar supplemented with 5 mg l⁻¹ MnSO₄ and with 1 mg l⁻¹ vitamin B₁₂, colonies are 1-2mm diameter, flat, circular, entire, beige or cream and smooth or glossy in appearance. They bear spherical to ellipsoidal endospores which lie in paracentral and subterminal, sometimes terminal, positions within slightly swollen and unswollen sporangia. Sporulation is infrequent but can be enhanced by using BHI-soil extract agar supplemented with vitamin B₁₂ and MnSO₄. Spores of strains isolated from UHT milk grow poorly and sporulate poorly, but their spores show very high heat resistance and have the ability to survive

204 ultra high temperature treatment (UHT). This very high heat resistance may decrease upon
 205 subculture. Isolates from farm environments may grow more readily than UHT milk isolates but be
 206 less heat resistant. Oxidase and catalase positive. Casein and starch are not hydrolysed. In the API
 207 20E strip: nitrate is reduced to nitrite, the Voges-Proskauer reaction is variable, citrate utilisation is
 208 variable, hydrogen sulphide and indole are not produced, and the ONPG reaction is negative.
 209 Gelatin and aesculin are hydrolysed, urea is not. Growth may occur between 20 and 55 °C, with an
 210 optimum of about 37 °C. Growth occurs between pH 5 and 9, and NaCl is tolerated up to 5% (w/v).
 211 In the API 50CHB gallery, acid without gas is produced from *N*-acetyl-glucosamine, D-glucose, D-
 212 fructose, maltose, and from sucrose and D-trehalose by most strains, but reactions may be weak.
 213 Acid production from the following carbohydrates is variable: amygdalin, arbutin, D-cellobiose,
 214 gentiobiose, glycerol, mannitol, D-mannose, D-melezitose, methyl-D-glucoside, salicin, starch
 215 (weak), D-tagatose, D-turanose and xylitol (weak). Acid is not produced from the following
 216 carbohydrates: adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-
 217 fucose, L-fucose, galactose, gentiobiose, gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate,
 218 glycogen, inulin, lactose, D-lyxose, D-melibiose, *meso*-inositol, methyl-D-mannoside, methyl-
 219 xyloside, D-raffinose, rhamnose, ribose, sorbitol, L-sorbose and D-xylose.
 220 The major cellular fatty acids (mean percentage \pm standard deviation of total fatty acids) after 24 h
 221 growth on BHI supplemented with vitamin B₁₂ at 37 °C are: iso-C_{15:0} (33.28 \pm 4.42), anteiso-C_{15:0}
 222 (24.87 \pm 2.32) and anteiso-C_{17:0} (19.58 \pm 3.45). The following fatty acids are present in smaller
 223 amounts (mean percentage \pm standard deviation of total fatty acids): C_{16:0} (6.69 \pm 2.32), iso-C_{16:0}
 224 (6.40 \pm 1.61), iso-C_{17:0} (5.29 \pm 1.22), iso-C_{14:0} (1.77 \pm 0.70) and C_{14:0} (1.51 \pm 0.81). The type strain
 225 has *meso*-diaminopimelic acid as the cell wall diagnostic diamino acid and contains the polar lipids
 226 diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, aminophospholipid and a
 227 phospholipid. Major menaquinones are MK7 and MK8. The G+C content is 36.7 mol% for the type
 228 strain LMG 17668^T (=LMG 17894^T (contains colony types t₁ and t₂), = DSM 10599^T, = M215^T, =
 229 MB 581^T) and 36.2 mol% and 36.5 mol% for strains LMG 19620 and LMG 17883, respectively;

230 the 16S rRNA gene sequence of the type strain is deposited at EMBL/GenBank under accession
231 numbers U49078 (type I), U49079 (type II) and U49080 (type III). In the variable characters listed
232 above, the type strain gives the following reactions: the Voges-Proskauer reaction is positive,
233 citrate utilisation is negative, and acid without gas is produced from arbutin, D-cellobiose, glycerol,
234 mannitol, D-melezitose, salicin, D-tagatose, D-turanose and xylitol, but not from amygdalin,
235 gentiobiose, D-mannose, methyl-D-glucoside and starch. Spores, though scanty, are ellipsoidal,
236 terminal, and do not swell the sporangia.

237 **Emended description of *Bacillus oleronius*.**

238 *Bacillus oleronius* (o.le.ro'ni.us, N. L. masc. adj. *oleronius*, pertaining to the island île d'Oléron
239 (France), the place where the organism was first isolated from the hindgut of a termite).
240 Cells are non-motile, Gram-negative, medium-sized rods, that occur singly and in pairs, and
241 sometimes form short chains of 3-4 cells. They bear ellipsoidal endospores that lie in subterminal
242 and paracentral positions within swollen sporangia. Spores do not show very high heat resistance.
243 After 2 d on TSA colonies are approximately 1-2 mm diameter, circular, entire, shiny, beige or
244 cream and butyrous with slightly translucent edges. Organisms are strictly aerobic and catalase
245 positive. Growth may occur between 30 and 50 °C, with an optimum of 37 °C. Growth occurs at pH
246 5.7 and at 6.8. NaCl is tolerated up to 7% (w/v). Casein is not hydrolysed and starch is sometimes
247 hydrolysed weakly. In the API 20E strip: nitrate is reduced to nitrite, the Voges-Proskauer reaction
248 is variable, citrate is not utilized, hydrogen sulphide and indole are not produced, and the ONPG
249 reaction is negative. Aesculin is hydrolysed, gelatin is weakly hydrolysed and urea is not
250 hydrolysed. In the API 50CHB gallery: acid without gas is produced from *N*-acetyl-glucosamine,
251 D-cellobiose, D-fructose, D-glucose, mannitol and D-tagatose. Acid production from the following
252 carbohydrates is variable, and when positive it is weak: galactose, glycerol, maltose, D-mannose,
253 ribose, salicin, starch and D-trehalose. Acid is not produced from the following carbohydrates:
254 adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, dulcitol, erythritol, D- and L-

255 fucose, gentiobiose, gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, methyl-D-glucoside,
 256 glycogen, *meso*-inositol, inulin, lactose, D- and L-lyxose, methyl-D-mannoside, D-melibiose, D-
 257 melezitose, rhamnose, D-raffinose, sorbitol, L-sorbose, sucrose, D-turanose, xylitol, D- and L-
 258 xylose and methyl-xyloside.
 259 The major cellular fatty acids (mean percentage \pm standard deviation of total fatty acids) after 24 h
 260 growth on BHI supplemented with vitamin B₁₂ at 37 °C are: iso-C_{15:0} (39.24 \pm 1.38), anteiso-C_{15:0}
 261 (22.89 \pm 2.22) and anteiso-C_{17:0} (20.78 \pm 0.85). The following fatty acids are present in smaller
 262 amounts (mean percentage \pm standard deviation of total fatty acids): iso-C_{17:0} (6.76 \pm 1.25), iso-
 263 C_{16:0} (5.37 \pm 0.50), C_{16:0} (3.35 \pm 0.29) and iso-C_{14:0} (1.09 \pm 0.15). The type strain has *meso*-
 264 diaminopimelic acid as the cell wall diagnostic diamino acid and contains the polar lipids
 265 diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, aminophospholipid and
 266 four different phospholipids. Major menaquinones are MK7 and MK8. The G+C content is 35.2
 267 mol% for the type strain LMG 17952^T (= DSM 9356^T, Rt 10^T) and 34.7 mol% for LMG 17886; the
 268 16S rRNA gene sequence of the type strain is deposited at EMBL/GenBank under accession
 269 number X82492. In the variable characters listed above the type strain is positive for the Voges-
 270 Proskauer reaction, and acid without gas is produced from: glycerol, maltose, ribose, starch (weak)
 271 and D-trehalose, but not from galactose, D-mannose or salicin.

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278

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Table 1: *B. sporothermodurans* and *B. oleronius* strains used and their origin.

Original species identification	Eventual new species identification	LMG number	R-number [*]	Other designations [†]	Source
<i>B. sporothermodurans</i>		17668 ^T	1948	LMG 17894 ^T (t ₁ and t ₂) ⁺ , M215 ^T , MB 581 ^T , DSM 10599 ^T	UHT-milk, Italy
<i>B. sporothermodurans</i>		17897	1951	MB 372	UHT-milk, Germany
<i>B. sporothermodurans</i>		18461		MB 512	UHT-milk, Belgium
<i>B. sporothermodurans</i>		18465		MB 373	UHT-milk, Germany
<i>B. sporothermodurans</i>			17269	MB 1320	sterilized milk, Dominican republic
<i>B. sporothermodurans</i>		17883	1937	MB 385	raw milk, Belgium
<i>B. sporothermodurans</i>		19637	3247	MB 1668	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6645	MB 1493	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6662	MB 1494	soy, Belgium
<i>B. sporothermodurans</i>			6665	MB 1495	soy, Belgium
<i>B. sporothermodurans</i>			6689	MB 1496	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6701	MB 1497	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6710	MB 1498	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6713	MB 1499	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6723	MB 1500	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6725	MB 1501	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6744	MB 1502	feed concentrate, Belgium
<i>B. sporothermodurans</i>			6777	MB 1503	soy, Belgium
<i>B. sporothermodurans</i>			6778	MB 1504	pulp, Belgium
<i>B. sporothermodurans</i>			6779	MB 1505	silage, Belgium
<i>B. sporothermodurans</i>			6786	MB 1506	feed concentrate, Belgium
<i>B. sporothermodurans</i>			7342	MB 1508	feed concentrate, Belgium
<i>B. sporothermodurans</i> <i>B. fordii</i>			7489	MB 1509	feed concentrate, Belgium
<i>B. sporothermodurans</i>		19617	3227	MB 1316	feed concentrate, Belgium
<i>B. sporothermodurans</i>		19620	3299	MB 1317	feed concentrate, Belgium
<i>B. oleronius</i>		17882	1936	MB 382	raw milk, Belgium
<i>B. oleronius</i>		17884	1938	MB 386	raw milk, Belgium

381	<i>B. oleronius</i>		17886	1940	MB 397	raw milk, Belgium
382	<i>B. oleronius</i>		17887	1941	MB 398	raw milk, Belgium
383	<i>B. oleronius</i>		17952 ^T		DSM 9356 ^T	termite
384	<i>B. oleronius</i>		19619	3297	MB 1318	feed concentrate, Belgium
385	<i>B. oleronius</i>			6450		milk installation, Belgium
386	<i>B. oleronius</i>			6691		feed concentrate, Belgium
387	<i>B. oleronius</i>			7770	MB 2102	milk installation, Belgium
388	<i>Bacillus</i> sp. [§]	<i>B. oleronius</i>	6724			feed concentrate, Belgium
389	<i>Bacillus</i> sp. [§]	<i>B. oleronius</i>	6464			filter cloth, Belgium
390	<i>Bacillus</i> sp. [§]	<i>B. oleronius</i>	6955		MB 2101	milk installation, Belgium
391	<i>Bacillus</i> sp. [§]	<i>B. oleronius</i>	6463			filter cloth, Belgium
392	<i>Bacillus</i> sp. [§]	<i>B. oleronius</i>	6504			milk installation, Belgium
393	<i>B. fordii</i>		22080 ^T	7190 ^T	DSM 16014 ^T	raw milk, Belgium
394					MB 1507 ^T	

395 *: R, Research collection of the LMG Bacteria Culture Collection; [†]: MB, collection of the molecular bacteriology lab of
396 the Institute of Agricultural & Fisheries Research (ILVO), Technology and Food Science Unit, Melle, Belgium; [‡]: colony
397 types; [§]: strains putatively identified as *B. oleronius* by FAME (Scheldeman *et al.*, 2004).

398 Table 2: DNA-DNA hybridisation values for *Bacillus sporothermodurans* strains and closest
 399 relatives.

			LMG 17668 ^T	LMG 18435 ^T	DSM 14745 ^T	LMG 17952 ^T
	<i>Bacillus sporothermodurans</i>	SDS-PAGE subgroup I	LMG 17668 ^T	100%		
			LMG 17833	81% [*]		
			LMG 19620	88% [*]		
		SDS-PAGE subgroup II	R-6778	83%		
		SDS-PAGE subgroup III	LMG 19637	76%		
	<i>Bacillus shackletonii</i>		LMG 18435 ^T	42%	100%	
	<i>Bacillus acidicola</i>		DSM 14745 ^T	35% [†]	25%	100%
	<i>Bacillus oleronius</i>		LMG 17952 ^T	16% [*]	ND	32% [†]
400	*.: data from Scheldeman <i>et al.</i> (2002); [†] : data from Albert <i>et al.</i> (2005)					

Table 3: Cellular fatty acid methyl ester profiles of *B. sporothermodurans*, *B. oleronius* and *B. fordii*. Presented values are averages for the number of strains analysed (mentioned between brackets) with the corresponding standard deviation.

Fatty acids	<i>B. sporothermodurans</i> (n = 24)	<i>B. oleronius</i> (n = 14)	<i>B. fordii</i> (n = 2)
Saturated fatty acids			
C _{14:0}	1.51 ± 0.81	< 1.00	< 1.00
C _{16:0}	6.69 ± 2.32	3.35 ± 0.29	2.65 ± 0.10
Branched fatty acids			
iso-C _{14:0}	1.77 ± 0.70	1.09 ± 0.15	< 1.00
iso-C _{15:0}	33.28 ± 4.42	39.24 ± 1.38	47.19 ± 0.86
anteiso-C _{15:0}	24.87 ± 2.32	22.89 ± 2.22	25.55 ± 5.49
iso-C _{16:0}	6.40 ± 1.61	5.37 ± 0.50	2.10 ± 1.44
iso-C _{17:0}	5.29 ± 1.22	6.76 ± 1.25	7.65 ± 4.19
anteiso-C _{17:0}	19.58 ± 3.45	20.78 ± 0.85	12.65 ± 2.12

Table 4. Some characters for distinguishing between *B. sporothermodurans*, *B. oleronius*, *B. fordii*, *B. acidicola*, *B. shackletonii* and some other weakly reacting species. All data were obtained in the course of this study or have been taken from other studies in the authors' laboratories. All characters were determined using tests in API 20E and API50CHB systems with the exception of microscope observations, starch and casein hydrolysis.

+, > 85% positive; (+), 75-84% positive; v, variable (26-74% positive); -, 0-15% positive; w, weak positive reaction; +/w, positive or weak positive reaction; v/w, variable and when positive the reaction is weak.

	<i>B. sporothermodurans</i>	<i>B. oleronius</i>	<i>B. rufis</i>	<i>B. circulans</i> T	<i>B. lentus</i> T	<i>B. firmus</i>	<i>B. fordii</i>	<i>B. acidicola</i>
Motility	v	-	+	+	+	+	+	+
Spores observed	rarely	+	+	+	+	+	+	+
Spores spherical	v	-	-	-	-	-	-	-
Sporangia swollen	v	+	+	+	-	-	v	+
Anaerobic growth	-	-	+	+	-	+	-	-
Gram reaction	+	-	+	+	+	+	-	+
Starch hydrolysis	-	v/w	+	+	+	+	-	-
Casein hydrolysis	-	-	-	w	-	+	-	-
API 20E tests								
ONPG reaction	-	-	+	+	+	-	-	-
Citrate utilisation (Simmons')	v	-	-	-	-	-	-	+
Urease production	-	-	-	-	+	-	-	-
Voges-Proskauer test	v	v/w	-	w	-	-	-	-
Gelatin hydrolysis	+	+/w	-	-	-	+	-	-
Nitrate reduction	+	+	+	+	+	v	-	+
API 50 CHB tests								
Glycerol	v	v/w	-	+	-	+	-	-
L-arabinose	-	-	+	+	w	-	-	-
Ribose	-	v/w	+	w	w	w	-	+
D-xylose	-	-	+	+	w	v	-	+
Methyl-xyloside	-	-	-	+	-	-	-	-
Galactose	-	v/w	v	+	w	-	-	+
D-mannose	v	v/w	+	+	+	-	-	w
Meso-inositol	-	-	-	+	w	-	-	-

mannitol	v	+	v	+	-	+	-	w
sorbitol	-	-	-	+	w	-	-	-
Methyl-D-glucoside	v	-	v	+	+	-	-	+
Amygdalin	v	-	-	+	w	-	-	+
Arbutin	v	-	-	+	w	-	-	+
Salicin	v	v/w	v	+	w	-	-	+
D-cellobiose	v	+	v	+	w	-	-	+
Maltose	+	v/w	v	+	w	+	-	+
Lactose	-	-	+	+	+	-	-	w
D-melibiose	-	-	+	+	w	-	-	+
Sucrose	(+)	-	+	+	+	+	-	+
D-trehalose	(+)	v/w	+	+	+	+	-	+
Inulin	-	-	v	+	-	-	-	+
D-melezitose	v	-	+	+	w	-	-	w
D-raffinose	-	-	v	+	w	-	-	+
Glycogen	-	-	v	+	-	+	-	+
Xylitol	v/w	-	-	+	-	-	-	w
Gentiobiose	v	-	-	+	w	-	-	+
D-turanose	v	-	-	+	w	-	-	-

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418 **Legend Supplementary figure.** Normalized computer profiles from SDS-PAGE analyses of whole
419 cell proteins of isolates belonging to the species *Bacillus sporothermodurans*, *B. oleronius* and *B.*
420 *fordii*. The dendrogram is based on UPGMA clustering of the correlation coefficient (r) of the total
421 protein profiles. The zone used for clustering is indicated in gray on the kDalton bar above the
422 profiles.
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